DEVELOPMENT OF AN INACTIVATED VACCINE AGAINST INFECTIOUS BURSAL DISEASE VIRUS

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PREFACE

This work was carried out in the Department of Microbiology, Faculty of Veterinary Medicine, University of Khartoum under supervision of Professor Abdelmalik Ibrahim Khalafalla.
DEDICATION

TO MY PARENTS
TO MY BROTHER
SHAZALI & SISTER SARA
TO HER AND THEM
AND FRIENDS
WITH MY DEEP LOVE
ACKNOWLEDGEMENT

First of all, my great thanks to almighty Allah who make this work possible and for giving me the health and strength to complete it.

I would like to express my gratefulness and gratitude to my supervisor Professor Abdelmalik Khalafalla for the tremendous help and guidance. The gratitude also due to Dr. Sobhi Ahmed Mohamed Kheir, Central Veterinary Research Laboratories for his personal interest and useful suggestions.

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My thanks and gratitude is extended to my colleagues and the staff of the Virology Research Laboratory; Dr. Sana Awad, Mr. Sharani Omer Musa, Mr. Abdelmonem Ramadan, Miss. Mawahib Awad and Mrs. Nadia.

My heartiest thanks are extended to Dr. Egbal Seid Ahmed at the Department of Avian Pathology and Diagnosis and Dr. Tamador at the Department of Virus Vaccine, Central Veterinary Research Laboratories and to every one who directly or indirectly participated in helping me to finish this work.
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## INTRODUCTION

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<td>IBDV</td>
<td>infectious bursal disease virus</td>
</tr>
<tr>
<td>VNT</td>
<td>virus neutralization test</td>
</tr>
<tr>
<td>ND</td>
<td>Newcastle Disease</td>
</tr>
<tr>
<td>VP</td>
<td>viral protein</td>
</tr>
<tr>
<td>Mab</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>VV</td>
<td>Very Virulent</td>
</tr>
<tr>
<td>Pi</td>
<td>post inoculation</td>
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<tr>
<td>AGID</td>
<td>Agar gel immunodiffusion</td>
</tr>
<tr>
<td>Ac-ELISA</td>
<td>antigen capture enzyme linked immunosorbant assay</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse – transcriptase/ polymerase chain reaction</td>
</tr>
<tr>
<td>CAM</td>
<td>chorioallantoic membrane</td>
</tr>
<tr>
<td>CEF</td>
<td>chick embryo fibroblast</td>
</tr>
<tr>
<td>SP</td>
<td>small plaque</td>
</tr>
<tr>
<td>LP</td>
<td>large plaque</td>
</tr>
<tr>
<td>AGPT</td>
<td>Agar gel precipitation test</td>
</tr>
<tr>
<td>CPE</td>
<td>cytopathic effect</td>
</tr>
<tr>
<td>ED</td>
<td>effected dose</td>
</tr>
<tr>
<td>MDA</td>
<td>maternal drivel antibody</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
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Abstract

In the present study an inactivated IBD vaccine were developed from a local field isolate of the virus. It is anticipated that the field virus would induce better immune response in vaccinated birds due to its close antigenic relation ship. A field outbreak of the disease that occurred in 2006 in Khartoum state (Shambat) was investigated that caused 70% mortality even in vaccinated bird. IBDV was detected by AGID test, isolation in cell culture (CEF) and reproduction of the disease in experimental chicks. The virus was isolated in CEF cell culture and identified serologically by AGDT. The virus was designed Shambat/06 and further propagated for two passages in cell culture and the TCID₅₀ was determined. Then the CEF cell culture harvest was inactivated using formalin and the virus inactivation was tested used experimental chicks.

A total of 140 layer chicks were used to determine the efficacy and safety of the developed vaccine and to compare it with commercial IBDV vaccines D78, Bur706.

The results showed that when the bursa suspension was inoculated onto chick embryo fibroblast cell culture it induce clear cytopathic effects after 5 days of inoculation. Clinical signs observed after experimental infection of susceptible chicks were similar to the classical IBD clinical signs and were characterized by sudden onset of depression, dullness, ruffled featherand death. Tissues collected from experimental chickens were examined histopathologically. Bursal of fabricius showed hemorrhage in the connective tissues and
the dissociated follicular cells beside edema in the follicle and degeneration of follicles. A volume of 100 ml of the inactivated virus was mixed with Aluminum hydroxide as adjuvant. Experimental chicks were vaccinated by the inactivated vaccine and for comparison additional chicks were vaccinated with D78 and Bur706 commercial IBD vaccines. No signs or lesions of IBD were seen and vaccinated chicks remained healthy throughout the experiment, which means that the IBDV in the vaccine was completely inactivated by the formalin.

Two preparations were made for the vaccine, with and without Aluminum hydroxide adjuvant and two routes of vaccination; intramuscular (I/M) and subcutaneous (S/C) were tried. The best result in terms of immune response and withstanding challenge was obtained when the adjuvanted inactivated vaccine was given by the intramuscular route. Results showed also that the developed vaccine without adjuvant when given by I/M gave a relatively lower protective immune response of 73.3% comparing with 93.3% for the adjuvanted vaccine which mean that the absent of the adjuvant make loss of 20% at immune response.

The results also revealed comparable protective immune response between D78, Bur706 vaccines and the developed adjuvanted vaccine when the latter was given by I/M route. On the other hand, the developed vaccine without adjuvant showed 20% lower protective immune response compared with the commercial vaccines. The results also showed similler withstanding challenge for both shambat/06/adj and D78 while Bur706 was slightly better than both above vaccines. The higher uniformed higher titer from 8,000
upto 12,000 was induced in vaccinated chicks by the developed vaccine with adjuvant when given by I/M.

The efficacy of the developed vaccine observed in the present study is promising and indicate that the developed vaccine may became a good alternative method to control IBD in Sudan.
ملخص الأطروحة

لقد أنجزت لجنة بحثية عامية ومحالية، أُنفرج عنها أثناء الدراسة هذه في التلفزيون، حيث أن بر我不是can'T．

 أنها، عام 2006، تحدت إلى متوسط معدل الموت الشهري في الحالات المصابة بحالة، حيث واجه بـ 70%، ونسبة تتعافى على الأرجح، والتدابير غير الشائعة في الانتشار، تتم على الأقل، وآخرون، ليس على الأرجح، لأنها، متغيرة، في الأمر.

 وقد، بعض الطرق، وتعزيز، العرض، في الفيروس، وتعويض، على بعض حالة، للصحة، وpostgresql، في هذه المعلومة.

 وتعويض بعض الحالات، في هذه المعلومة.

 وتعويض بعض حالة، للصحة، وpostgresql، في هذه المعلومة.
عöz àæsâçî
dëkôdûj oû âr jëzdàg le
Nëj jëzdû oû ñësû
gîa àâxô àf këfîf hìzo ñëzà s ñësî hëkët yëldûjëf õsî jëzdû
nekhàkàf dëu ñës ñëzûdûjëf qëf së t ñëf sëÎû ñûj ñëzûdûjëf
yjàn gënsûniq õnî ñëf ñëpëdëf ñp ñësàg tàû jëzdûj

. Jëzdû Kût âo õs îdëg áe këfûgës tónë
yâu à fësûđûf ñû àærûniûdëfûj yëzdàg s ñësî jëzdû 00 ñëzûdû
ljö dëlûlëfà (Kêmê/06) ñjâ fënhûr këfà ljö dëlûr fënhûlûntëf
fâu ad këfûqës ñû dënhûkàz Bur706, D 78 Kêmê ñhû fû fâhûkàz

. Nëjû õzûpûgëdûfûj ñû këfût ñ ñhûlûfûhûlûj
yâdëf ñû àærûniûdëfûj ñû ñësûkôf ñëkëntûs Kêmê/06) ëf ñëf sëfûzû
ôdûkàf ñû fûfîj ljö dëkëf. ñû àærûniûdëfûj ñû ñëfûsû ljëk spûd ñëkëf yëzdû
fû àf ñësûkjû ñû këfûjû klû ñûf ûyûr zëf õsà (ñëfû kûl jëfôf ñfûpàr Kûmê/06)
gënlû dëkî ñf ñûdkëfûj 5ljëk ñûfûlûfûj ëfûf së fënhûkàzûnëkûfûj ñûf
le ñ uzdû ñûf ñûkëfûjû yëdzû ngëfûsû õsà (Kêmê/06) ñëzûdûkàf ñû fûfîj
êfû jëfû ñû dënhûkàf ñû ñûdkëfûjû yëdzû õsà ñûdûkàf Kûl jëfû ñûfûlûfûj 3û õsà
2 ñûdkëfûjû dënhûkàz ñûfûlûfûj 3û õsà le ngëfû dënhûkàf yëdzû

. ñûdkëfûjû ñûfûlûfûj õsûjû ñûkëfûjû yëdzû tónë ñûgûnlûfûjû yëdzû õsà

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إعداد تقرير صحي

النتائج

التجارة الفاصية بين مكانية

Bur 706 , D78 مع الفاصية (K07/06) لـ D 78 (K07/06) ليس له دور كبير في ظهور الصعوبات

. لذا للجية فإن الجسم والبشرة تدمر جزء من Bur 706 لصغر حجم

إلى 8,000) لعينات مرتدة تكون نتيجة تقارن للساقاك لتشمل

(K07/06) بعثت دورة الأشجار لمساكن النبات لدراسة حجم 12,000

. يعرف أن بعض الأشجار يمكن أن تكون مبتورًا من الأجزاء

فيديف دون أن تكون على النبات في الرغم من وجود بعض النباتات

التي لا تضر بالنبات أو الأجزاء الأولى من الأدوار المركبة 

. لذا
Introduction

Infectious bursa disease (IBD) an acute viral disease of young chickens was first described by Cosgrove (1962), characterized by destruction of the bursa of Fabricius followed by immunosuppressant (Allan, Faragher and Cullen, 1972, Fadley, Winterfield and Gander, 1976). The disease was widespread in chickens and is of great economic importance for both broiler and pullet growers (Lukert and Saif, 1991).

The virus genome consists of two segments of double strand RNA. Based on virus neutralization test (VNT) IBDV is classified into serotypes, serotype 1 and 2 (Jackwood, 1985). Serotype 1 viruses are pathogenic to chickens while serotype 2 viruses (isolated from turkeys) are non-pathogenic to chickens.

Recently, the IBDV has become more virulent and the picture of the disease has changed and become more severe than in early outbreaks in 1980s. In the recent years IBD become the most devastating disease of chicken in the Sudan with mortality rates that exceed 60% even in vaccinated flocks, with the ability of the virus to change its genetic structure in order to survive a continual barrage of new vaccines.

The vaccine may not contain the proper strains or serotypes of organism required to stimulate protective immunity against the field virus. Although the vaccine is administered properly and uniformly and adequate antibody titers are present, the chickens still break with the disease.
The objectives of this study are
1. To isolate IBD field strain virus
2. To develop a vaccine by propagation and inactivation of the isolated virus.
3. To compare the immune response of the developed inactivation local vaccine with commercial vaccines (D78 and Bur706).
Chapter One
Literature Review

1.1. Infectious Bursal Disease

1.1.1. Definition

Infectious bursal disease is an acute disease of young chickens of 3-6 weeks old, characterized by severe depression, ruffled feather, whitish watery diarrhea, anorexia, depression, occasionally nephrosis, dehydration and death due to destruction of lymphoid cells in the bursal of Fabricius and other lymphoid organs (Anon, 2000).

1.1.2. History

IBD was discovered by Cosgrove in 1962, at that time it was called avian nephrosis because of the extreme kidney damage found in birds that succumbed to infection. The disease was first observed in Gumboro, Delaware, USA by Cosgrove (1962) as a result the disease is often referred to as (Gumboro). Winterfield and Hitchner (1962) later isolated the causative virus and differentiated the IBD entity from the nephritis syndrome caused by certain infectious bronchitis variant viruses, the isolate was identified as infectious bursal disease (IBD), as the disease causes specific pathognomic lesions in the cloacal bursa.

1.1.3. Economic importance

The economic impact of IBD is influenced by the strain of the virus, susceptibility of flocks, intercurrent primary and secondary environmental and managemental factors, flock livability, weight gain, conversion and reproductive efficiency (Shane et al., 1994).
In addition to deaths, and immunosuppression, losses from IBD including depressed growth rate, feed conversion efficiency are recorded in affected broiler flocks (Shane et al., 1994).

Furthermore the increase use of antibiotics and chemicals to fight secondary infections is a major concern of human health, if we consider the risks linked to the presence or residues in meat products, the release of residues into environment and increased antibiotic resistance (Marian, 2001).

Monitoring is vital studies that show economic impact of both clinical and subclinical IBDV infections can be significant. It is also too easy to underestimate the problem. Monitoring is an important tool to help to minimise the damage. Besides, the aforementioned reasons (a subclinical infection is a risk factor for a clinical outbreak in the next flock, and can cause immunosuppression, which may result in significant economic losses), monitoring also provides information about vaccine application. When vaccinated birds are still negative in IBDV serology at slaughter this is a strong indication that the vaccination protocol needs to be checked. It is better to carry out checks on a regular basis, rather than when there are complaints, because by then it is already too late (Sjaak, 2001).

1.1.4. IBDV in Sudan

The first outbreak in the Sudan was observed at El Obied (North Kordofan state) in 1981 with pronounced mortality (36%) (Shuaib et al., 1982). Since that time the disease has been reported in many parts of the Sudan and became a serious problem facing the poultry
industry in the Sudan (Hajer and Ismail, 1987). (Gaffer et al., 1987) found that Kassala State sera epidemic logically related virus strain to that of Elobied outbreak was reported. Then in Nyala in 1986 with 50% mortality and another outbreaks in the same location with 45% mortality (Mansour et al., 1993) and since then it became endemic whenever massive poultry production is practiced in the country (Shuaib et al.,1982., Salman et al.,1983 and Mahasin1998). Although El Hussein et al.,(1998) observed some seasonal in northern Sudan. The recorded symptoms were likely confined to the exotic breeds (Hajer et al., 1988 and Mansour et al., 1993). However, surveys revealed presence of specific antibodies against IBD virus in local types of chickens yet they had not been vaccinated and seem apparently healthy (Elhassan et al., 1989) and Gaffar Elamin et al., 1987). A mild form of the disease in broilers was reported by Khalafalla et al., (1990)

Incidence of sub clinical IBD infection in Sudan was evidently discussed by, Mahasin (1998). She attributed the vaccination failure-that is usually encountered-to these infections.

Unfortunately, vaccination of birds irrespective to their status of immunity or the type of vaccine is usually practiced by many breeders (personal communication). Moreover, the highly variable IBD vaccines which including invasive strain were frankly used in routine vaccination programmer, despite that their use – in some countries-necessitate previous approval form the authorities Gardin (1994).
1.2. Aetiology

1.2.1. Classification and structure

At the first time IBDV was classified as a picorna virus (Lunger and Madux 1972). Then as Reo virus (Petex and Mandelli, 1968; Koster et al., 1972). At last IBDV was regarded as a member of Birnaviridae family in the Avibirnavirus genus. Other genera in that family were Aquabirnavirus of pancreatic necrosis virus of fish (Murphy, Gibbis, Horzinek and Student 1999). The genome consists of two double stranded RNA segments designated as A and B (Miiler et al., 1979). The smaller genomic segment encodes viral protein (VP1) the viral polymerase (Azad, Barret and Fahey, 1985). The larger segment of the genome encodes three proteins VP2, VP3 and VP4, of which VP2 are structural proteins, also VP2 exists as precursor protein (VPx) and cleaved product, VP2 neutralizing monoclonal antibodies (Mab) have been shown to bind to VP2 whereas VP3 does not carry neutralizing epitopes (Fahey et al., 1991 and Snyder et al., 1992). The VP3 is regarded as a group specific antigen as it is recognized by monoclonal antibodies directed against VP3 from strains of both serotype 1 and 2 IBDV (Becht et al., 1988), while VP4 is viral protease (Fahey et al., 1991). VP4 and VP5 respectively are responsible for the maturation of capsid proteins and for virus releases from the infected cell (Eterradossi, 2001).

1.2.2. IBDV serotypes

Two serotypes of IBDV had been recognized known as serotype 1 and serotype 2 (Jack Wood, Saif and Huges, 1982, Mcferran et al., 1980). The two serotypes are differentiated by virus neutralization
(VN) test (Lukert and Saif, 1991). Only serotype 1 viruses are pathogenic for chickens while serotype 2 is most prevalent in Turkeys (Ismail, Saif and Moorhead, 1988), also serotype 2 strain neither cause disease nor protection against infection with the serotype 1 strains (Katizierenberg et al., 2004). The two serotypes share a common antigen demonstrated in agar gel precipitation test (Synder et al., 1992).

1.2.3. IBDV serotype 1 strains

The terms (variant), (classical), (very virulent) have been used to qualify the IBDV stains that exhibit a different pathogenicity. North American variants IBDVs include little if not clinical signs and no mortality but marked bursal lesion. Classical IBDVs induce approximately 10-50% mortality with typical signs and lesions where very virulent IBDVs induce approximately 50-100% mortality with typical signs and lesions (Eterradossi et al., 2001).

Jack Wood and Saif (1987) using (VN) test, detected significant antigenic differences among serotype 1 IBDV. They studied 8 serotype 1 commercial vaccine strains, 5 serotype 1 field strain and 2 serotype 2 field strains. Six subtypes were distinguished among the 13 serotypes. One of the subtypes included all of the variant isolates (Saif et al., 1987). These subtype viruses are causing infection often before 2 weeks of age with no mortality but bursal atrophy (Vob and Vielitz, 1994).

1.2.4. Very virulent (VV) IBDV strain

In 1987 a highly pathogenic strain (84VB) of type 1 IBDV emerged in Holland and Belgium (Van den Berg et al., 1991).
Mortality in layer replacement pullets attained 70% and 100% in experimental infection. These strains break through maternal antibodies, which were protective against classical strains (Chettle et al., 1989, Vob and Vielitz, 1994). Within few years, this highly pathogenic IBDV strain spread over Europe, Middle East, South Africa and South East Asia (Vob and Vielitz, 1994). In the Sudan the highly virulent strains were detected within the viruses isolated since 1994 (onwards, Mahasin, 2001).

1.3. Pathology of IBD

1.3.1. Susceptible age

Clinical IBD is most commonly recognized in susceptible 3-6 weeks old birds, chickens younger than 3 weeks have subclinical infection (Lukert and Saif., 1991). Light layer breeds have been reported to be more susceptible than heavy broilers (Van den Berg and Meulemans., 1991).

1.3.2. Transmission of IBDV

IBD is excreted in the feces of infected birds for 2-14 days; it is highly contagious and transmission occurs directly through contact and oral uptake (Murphy et al., 1999). IBD is stable at pH 3 to pH 9 and can survive 60°C for 60 minute (Murphy et al., 1999). Due to the hardy nature of the virus it persists in the environment of the poultry house. Infections are thus potentially carried over from one cycle to the next. It can be transmitted from infected hens to others by contaminated tools, equipment, feed or water, boots or clothes of labors and all people who move from farm to other. The *Aedes vexans* mosquitos and meal worms (*Alphitobius diaperinus*), have been
incriminated as even more important vectors (Howie and Thorsen, 1981, Snedeker, Wills and Moulthrop, 1967). IBDV was demonstrated in tissue sample of rat’s coat dead in a house that has a history of IBDV (Lasher and Shane., 1994). Vertical transmission probably occurs via eggs (Murphy et al., 1999). Torents et al (2004) have studied the possibility of transmission of IBDV by dogs. A single Beagle dog feed chicks infected by very virulent strain of IBD. After-ward the presence and the viability of IBDV was detected in dog feces for 2 days after initial ingestion. Techniques of the administered and excreted IBDV show similar characters between the two viruses (Torents et al., 2004).

**1.3.3. Pathogenicity of IBDV**

The target organ of the IBDV is the bursa of Fabricius which is specific reservoir of B lymphocyte in avian species. Bursectomy can prevent illness in chicks infected with virulent virus (Hiragar et al., 1994; Murphy et al., 1999). The severity of the disease is directly related to the number of susceptible cells present in the bursa of Fabricius. Therefore the highest age of susceptibility is between 3-6 weeks, when the bursa of Fabricius at its maximum development. After oral infection or inhalation, the virus replicates primary in the lymphocyte and macrophage of gut associated tissue. Then virus travels to the bursa via blood stream, where replication occurs. By 13 hours most follicles are positive for virus and by 16 hours post infection, a second and pronounced viraemia occurs in other organ leading to disease and death (Miiller et al., 1979). No disease occur
after 15-16 weeks of age when the bursa is regressed (Sainsbury, 2003).

The terms variant, classical, very virulent, have been used to qualify the IBDV strains that exhibit a different pathogenicity.

### 1.3.4. Histopathology

The spleen had hyperplasia of reticuloendothelial cells around the adenoid sheath in arteries in early stages of infection. By the third day there was lymphoid necrosis in the germinal follicles and periarteriolar lymphoid sheath. The spleen recovered from infection rather rapidly, with no sustained damage to germinal (Lucent and Saif, 1991). The thymus and caecal tonsils exhibited some cellular reaction in the lymphoid tissues in early stages of infection, but as in the spleen the damage was less extensive than in bursa and recovery was more rapid. Harderian gland was severely affected by infection of one-day old chicks with IBDV, which results in reduced infiltration of plasma cells (Dohms, and Rosenberger, 1981). Histological lesions of the kidney are non-specific (Peters, 1967), and probably occur because of severe dehydration of affected chickens. Helmboldt and Garner (1964) found kidney lesions in less than 5% of birds examined. Lesions observed were large casts of homogeneous material infiltrated with heterophils.

### 1.3.5. Morbidity and Mortality

Morbidity and mortality depend on the virulence of the challenged virus, the immune status and age of the infected birds and other factors affecting the pathogenicity of IBDV in full susceptible flocks, there is high morbidity rate usually approaching 100% (Lukert
and Saif, 1991). Classical mortality ranges from zero to 30 % (Lukert and Saif, 1991), but very virulent IBDVs strains can cause mortality of 70%-80% (Chettle et al., 1989, Murphy et al., 1999).

1.4. Immunosuppression

The term immunosuppression is defined as a state of temporary or permanent dysfunction of the immune response resulting from insult to the immune system and leading to increased susceptibility to disease (Dohms and Saif, 1983). IBD immunosuppression first recognized by Allan, Faragher and Cullen (1972) and Faragher et al (1974). It is due to market depletion of lymphocytes in the lymphoid organs (Nakamura et al., 1992). Immunosuppression resulting from IBD infection, weakness response to vaccination and makes chickens more susceptible to a number of other infections diseases (Anon, 2000).

The main target cells for IBDV replication are the activity dividing B cell; thus infection leads to the destruction of B cells in the bursa of Fabricius, the primary organ of B cell development, and to lesser degrees in other organs such as the caecal tonsils, and spleen. The destructive effect of IBDV on B cells leads to a dramatic reduction in the ability of the IBD infected bird to produce antibodies against antigens, in addition to reducing humeral immunity. IBDV also leads to suppression of the cell mediated immune response, also suppression of macrophage function (Rautens Chlein, 2001).

1.5. Diagnosis of IBD

Diagnosis of IBD in chickens is historically based on clinical signs, gross lesions, and histopathology. Confirmation of the
diagnoses is based on detection of viral antigen, detection of antibodies and isolation of the virus (Liiticken, Vanloon and Devries, 1994).

1.5.1. Isolation of IBDV in chick embryo

The chorioallantoic membrane (CAM) of 9-10 day old embryo was the most sensitive route for isolation of the virus (Hitchner, 1970). Injection of the virus into 10-day old embryonated eggs resulted in embryo mortality from 3-5 days past inoculation. According to Winterfield, (1969) the virus increased in allantoamniotic fluid by serial passage in embryonating eggs, while Okoge (1984) mentioned that serial transfer of IBDV in chick embryo may reduce virulence for chickens. The recent very virulent strains are difficult to cultivate in embryonated eggs (Bumstead et al., 1993).

1.5.2. Isolation of the virus in chickens

This method has been used in the past but is no longer recommended due to animal welfare concerns. Five susceptible and five IBD-immune chickens (3–7 weeks of age) are inoculated by the eye drop route with 0.05 ml of sample. The bursa of chickens infected with virulent serotype 1 IBDV appears yellowish (sometimes hemorrhagic) and turgid, with prominent striations. Peribursal oedema is sometimes present, and plugs of caseous material are occasionally found. The presence of lesions in the bursa of susceptible chickens along with the absence of lesions in immune chickens is diagnostic of IBD. The bursa from both groups may be used as antigen in an agar gel immunodiffusion (AGID) test against known positive IBD antiserum. The extent of bursal damage may
vary considerably with the pathogenicity of the studied IBDV strain. However, as the samples submitted for virus isolation may vary in virus content, the extent of bursal damage observed in susceptible chickens at the isolation stage gives a limited indication on strain pathogenicity. The bursa of chickens infected with serotype 2 IBDV does not exhibit any gross lesions.

1.5.3. Isolation of the IBDV in cell culture

Many strains of IBDV have been adapted to cell cultures of chicken embryo origin and cytopathic effect has been observed. Cell culture adapted virus may be quantified by plaque assay (Lukert and Saif, 1991). The virulent IBDV are very difficult to adapt to tissue culture and when adapted became less virulent (Mekkes and Deuite, 1994). IBDV grows in chicken embryo fibroblast and produces (CPE) characterized with and appearance of round retractile cells in about 3-5 days (Sivanadan et al., 1993). The old strains or classical strain of IBDV can be isolated in chicken embryo kidney (CEK) (Lukert and Davis, 1974), also mammalian continuous cell lines known to be susceptible to IBDV (RK-13) derived from rabbit kidneys (Peter, Daprile and Cancellotti, 1973), Vero cells derived from African green monkey kidneys (Jack Wood et al., 1987), and MA-104 cell from fetal monkey kidneys (Jack Wood et al., 1987). Wild type IBDV strains particularly very virulent strain do not grow in tissue culture. Comparison of genome sequence of wild type and tissue culture adapted IBDV strains pointed to several mutations that might be responsible for invtro growth of IBDV in tissue culture (Islam, 2002). When the replication of IBDV in Vero cells and chicken
embryo kidney cells was compared no differences in virus titres were observed (Leonard, 1974). However, the replication cycle was found to be longer in Vero cells than in CEF cells (Lukert et al., 1975).

1.5.4. Plaque production

Plaque formation of IBDV growth in cell culture was reported (cho et al., 1979) in two shape small plaque (SP) and large plaque (LP) variants were obtained from IBDV adapted to chick embryo cell culture (Saijo, Higashihara, Fujisaki and Matumoto, 1990).

1.6. Identification of the agent

Clinical IBD has clearly characteristic signs and post-mortem lesions. A flock will show very high morbidity with severe depression in most birds lasting for 5–7 days. Mortality rises sharply for 2 days then declines rapidly over the next 2–3 days. Usually between 5% and 10% of birds die, but mortality can reach 30–40%. The main clinical signs are watery diarrhea, ruffled feathers, reluctance to move, anorexia, trembling and prostration. Post-mortem lesions include dehydration of the muscles with numerous ecchymotic hemorrhages, enlargement and orange discoloration of the kidneys, with urates in the tubules. The bursa of Fabricius show the main diagnostic lesions. In birds that die at the peak of the disease outbreak, the bursa is enlarged and turgid with a pale yellow discoloration. Intrafollicular hemorrhages may be present and, in some cases, the bursa may be completely hemorrhagic giving the appearance of a black cherry. Peribursal straw-coloured oedema will be present in many bursas. Confirmation of clinical disease or detection of subclinical disease is best done by using immunological
methods as the IBDV is difficult to isolate. Differentiation between serotypes 1 and 2 or between serotype 1 subtypes or pathotypes should be undertaken by a specialized laboratory (OIE, 2004).

1.6.1. **Sample preparation**

Remove the bursa of Fabricius aseptically from approximately five affected chickens in the early stages of the disease. Chop the bursa using two scalpels, add a small amount of peptone broth containing penicillin and streptomycin (1000 µg/ml each), and homogenize in a tissue blender. The homogenate should be centrifuged at 3000 g for 10 minutes and the supernatant fluid was collected in sterile vials for use in the virus investigations. Filtration through a 0.22 µl filter may prove necessary to further control bacterial contamination, although this may cause a reduction in virus titer (OIE, 2004).

1.6.2. **Identification by the agar gel immunodiffusion test**

The AGID is the most useful of serological test for detection of specific antibodies in serum or for detecting viral antigen in bursal tissue (OIE, 2004). The test is specific because it can not give false positive results, but it can give a false negative result. The presence of IBDV antigen can be detected in the bursal tissue by AGID for 5-6 days Pi (Murphy et al., 1999).

1.6.3. **Virus Neutralization Test (VNT)**

VNT is carried out in cell culture. The test is more laborious and expensive than the AGID, but is more sensitive for detecting antibody. The sensitivity is not required for routine diagnostic purpose, but may be useful for evaluation vaccine responses or for
differentiation between IBDV 1 and 2 serotypes (OIE, 2004). It is
difficult to use VNT in recent vvIBDV strains, because they are
difficult to cultivate in cell culture.

1.6.4. **Identification by antigen-capture enzyme-linked immunosorbent assay (AC-ELISA).**

Different protocols have been described for the detection of
serotype 1 IBDV using an antigen-capture enzyme-linked
immunosorbent assay (AC-ELISA). Briefly, ELISA plates are coated
with IBDV-specific antibodies. Depending on the chosen AC-ELISA
protocol, the capture antibody may be a mouse anti-IBDV
monoclonal antibody (MAb), or a mix of such MAbs, or a chicken
post-infectious anti-IBDV polyclonal serum. Unbound antigens are
discarded at the end of the incubation period by washing with a
suitable washing buffer. The captured antigens are then revealed, as
in an indirect ELISA, with a detection antibody, followed by an
enzyme conjugate that binds to the detection antibody only, followed
by the enzyme substrate. Finally, optical densities, which parallel the
amount of captured IBDV antigens, are read with an ELISA reader
(Marquardt et al., 1980)

1.6.5. **Identification by molecular techniques**

Molecular virological techniques have been developed that
allow IBDV to be identified more quickly than by virus isolation. The
most frequently used molecular method is the detection of IBDV
genome by the reverse-transcription polymerase chain reaction (RT-
PCR). This method can detect the genome of IBDV, which is unable
to grow in cell culture, because it is not necessary to grow the virus
before amplification. RT-PCR is performed in three steps: extraction of nucleic acids from the studied sample, reverse transcription (RT) of IBDV RNA into cDNA, and amplification of the resulting cDNA by PCR. The two latter steps require that the user selects oligonucleotide primers that are short sequences complementary to the virus-specific nucleotidic sequence. Different areas of the genome can be amplified depending on the location from which the primers have been selected.

1.7. Vaccines

The term ‘vaccine’ is used for products containing live or inactivated viruses or protozoa, live bacteria, or nucleic acids. Products containing killed bacteria and other microorganisms are identified as bacterins, bacterial extracts, subunits, bacterintoxoids, or toxoids, depending on the type of antigen they contain, products administered to animals in order to produce active or passive immunity or to diagnose the state of immunity’, however, the term ‘vaccine’ will include all products designed to stimulate active immunization of animals against disease

1.7.1. Vaccine type

1.7.1.1. Live vaccine

Live IBD vaccines are produced from fully or partially attenuated strains of virus, known as ‘mild’, ‘intermediate’, or ‘intermediate plus’ (‘hot’), respectively. Mild or intermediate vaccines are used in parent chickens to produce a primary response prior to vaccination near to point-of-lay using inactivated vaccine. They are susceptible to the effect of MDA so should be administered only after all MDA has
waned. Application is by means of intramuscular injection, spray or in the drinking water, usually at 8 weeks of age (Skeeles et al., 1979). Intermediate or intermediate plus vaccines are used to protect broiler chickens and commercial layer replacements. Some of these vaccines are also used in young parent chickens if there is a high risk of natural infection with virulent IBD. Although intermediate vaccines are susceptible to the presence of MDA, they are sometimes administered at 1-day old, as a coarse spray, to protect any chickens in the flock that may have no or only minimal levels of MDA (Mazariegos et al., 1990). This also establishes a reservoir of vaccine virus within the flock that allows lateral transmission to other chickens when their MDA decays. Second and third applications are usually administered, especially when there is a high risk of exposure to virulent forms of the disease or when the vaccinated chicks exhibit uneven MDA levels (Kouwenhoven, et al., 1994). The timing of additional applications will depend on the antibody titers of the parent birds at the time the eggs were laid. As a guide, the second dose is usually given at 10–14 days of age when about 10% of the flock is susceptible to IBD and the third dose 7–10 days later. Live IBD vaccines are generally regarded as compatible with other avian vaccines. However, it is possible that IBD vaccines that cause bursal damage could interfere with the response to other vaccines (OIE, 2004).

1.7.1.2. Inactivated vaccine

Inactivated IBD vaccines are used to produce high, long-lasting and uniform levels of antibodies in breeding hens that have previously been primed by live vaccine or by natural exposure to field
virus during rearing (Cullen and Wyeth, 1975). The usual programme is to administer the live vaccine at about 8 weeks of age. This is followed by the inactivated vaccine at 16–20 weeks of age. The inactivated vaccine is manufactured as a water-in-oil emulsion, and has to be injected into each bird. The preferred routes are intramuscular into the leg muscle, avoiding proximity to joints, tendons or major blood vessels or the subcutaneous route. A multidose syringe may be used. Used in this way the vaccine should produce such a good antibody response that chickens hatched from those parents will have passive protection against IBD for up to about 30 days of age (Wyeth and Cullen, 1979). This covers the period of greatest susceptibility to the disease and prevents bursal damage at the time when this could cause immunosuppression.

1.7.1.3. In Ovo vaccine

Recently, technology has been developed to deliver live vaccine into eggs during the incubation period. Live vaccine virus is blended with IBD antibody and the complex is injected in ovo at 18 days of incubation. The eggs go on to hatch and the vaccine virus is released when the chicks are about 7 days of age. In this way, the problem of maternally derived IBD antibody is overcome and the chicks are effectively immunized (Haddad et al., 1997).

1.7.1.4. Recombinant vaccines

The concept of recombinant vaccines is to insert genes of critical immunizing epitopes of a disease agent into nonessential gene of a vector virus. Vaccination with the recombinant virus thus results in immunization against both the vector virus as well as the expressed
epitopes of the disease agent (Hung et al., 2004) have developed a recombinant Newcastle disease virus (NDV) expressing VP2 protein of IBDV that protect against both NDV and IBD.

1.8. Vaccine manufacture

The vaccine must be manufactured in suitable clean and secure accommodation, well separated from diagnostic facilities or commercial poultry. Production of the vaccine should be on a seed-lot system using a suitable strain of virus of known origin and passage history. Specific progeny free (SPF) eggs must be used for all materials employed in propagation and testing of the vaccine. Live vaccines are made by growth in eggs or cell cultures. Inactivated IBD vaccines may be made using virulent virus grown in the bursae of young birds, or using attenuated, laboratory-adapted strains of IBDV grown in cell culture or embryonated eggs. A high virus concentration is required. These vaccines are made as water-in-oil emulsions. A typical formulation is to use 80% mineral oil to 20% suspension of bursal material in water, with suitable emulsifying agents. (FAO).

1.9. Advantages and Disadvantages of live vaccines

Advantages of live-type vaccines are ease of administration, low price, rapid onset of immunity, and a broader scope of protection because chickens are exposed to all stages of the replicating virus. Disadvantages include problems with uniform vaccine application, excessive vaccine reactions, unwanted spread of the vaccine virus to neighboring poultry houses, and extreme handling requirements needed to maintain viability of the vaccine organism.
1.10. Advantages and Disadvantages of killed vaccines

Advantages of killed-type vaccines are assurance of administration of a uniform dose (birds are individually injected), safety (the organism has been inactivated), development of uniform levels of immunity (each bird receives the same dose), no chance for spread of vaccine organism to neighboring poultry farms, increased product stability, and a choice of a wider variety of virus strains. Disadvantages are increased costs (labor and product), slower onset of immunity, narrower spectrum of protection, and presence of localized tissue damage at site of injection due to reaction with the adjuvant.

1.11. Vaccination

Selecting a vaccination program for broilers and commercial layers involves the following considerations:
1. Size and scope of the operation. Operation of single or multiple-age facilities.
3. Pathogenicity of IBD viruses to which flocks may be exposed.
4. Availability of live attenuated (intermediate-plus or "hot") and inactivated vaccines

Source of chicks, whether from company-operated parent flocks or from purchased commercial eggs or chicks. (OIE, 2004).

1.12. Administration

The route of administration is by means of spray or in the drinking water. Intramuscular injection is used rarely. If the vaccine
is given in the drinking water, clean water with a neutral pH must be used that is free from smell or taste of chlorine or metals. Skimmed milk powder may be added at a rate of 2 g per liter. Care must be taken to ensure that all birds receive their dose of vaccine. To this end, all water should be removed (cut off) for 2–3 hours before the medicated water is made available and care must be taken that no residual water remains in the water adduction pipes or in the drinkers. It is possible to divide the medicated water into two parts, giving the second part 30 minutes after the first. (OIE, 2004)

1.13. Vaccine Failure

Multiplication of live vaccines, reducing the amount of immunity produced. For example, if a chick comes from a breeder hen with high levels of antibody against Gumboro, the chick will typically have high levels of antibodies. A high level of maternal antibodies in the young chicken may interfere with the (maternal) for several weeks if vaccination is attempted in the presence of these antibodies (Van den Berg et at., 1991).

Stress may reduce the chicken's ability to mount an immune response. Stress could include environmental extremes (temperature, relative humidity), inadequate nutrition, parasitism, and other diseases. Chickens should not be vaccinated during periods of stress. That is, delay vaccination until the birds are healthy.

Live vaccines may be inactivated due to improper handling or administration. Before administering live vaccines, check and record lot numbers and expiration dates on the vials. Store and handle vaccines as recommended by the manufacturer the vaccine may not
contain the proper strains or serotypes of organism required to stimulate protective immunity. Although the vaccine is administered properly and uniform and adequate antibody titers are present, the chickens still break with the disease, particularly with infectious bronchitis and more recently with infectious bursal disease.

Poor distribution of live vaccine administered by the water or spray route may result in chickens being "missed" in parts of the house (Relying on transmission of the vaccine from bird to bird is risky, and can result in excessive rolling-type reactions of long duration and delayed immunity in the flock [Farmer, 1992]. "Misses" with killed vaccines will result in chickens with no protection, as killed vaccines will not spread from bird to bird.

Chickens may already be incubating the disease at the time of vaccine administration. Despite proper administration, the birds become diseased because time is needed for antibody production to begin and reach protective levels. Remember, after first exposure to a live virus-type vaccine, immunoglobulin's G, M, and are first detected approximately 4 to 5 days following exposure. Additional days are required for titers to reach protective levels.

### 1.14. IBDV control

The control of IBDV strains involves many factors, such as disinfection, vaccination programs and understanding the epidemiology of the IBDV. IBDV like all viruses have known treatment, also IBDV have high stability in the environment, therefore IBDV can persist in poultry houses after cleaning and disinfection. The principle method to control the disease is therefore
by vaccination (Van Denberg and Meulemans, 1991). High levels of biosecurity should be enforced with specific reference to movement of personnel, feed and vehicles. On-farm trading and prolonged depletion periods will result in the introduction and persistence of vvIBD infection. It is essential to operate farms on an all-in-all-out cycle and to maintain an acceptable distance between units to prevent cross transmission of vvIBDV.

It is necessary to maintain high and uniform levels of protective maternal antibody in commercial chicks. This is achieved by effective priming and boosting the immune system of parent flocks placed on isolated farms. Vaccination programs should incorporate successive intermediate strain vaccines or in extreme risk situations, intermediate-plus vaccine followed by subsequent administration of inactivated bursal-derived emulsion vaccines. Maternal antibody levels should be monitored using an ELISA or quantitative agar gel procedure to measure the efficacy of the vaccination program (Gardin, 1994). Broiler chicks, under extreme risk, should be vaccinated with intermediate-plus ("hot") vaccine. If this unobtainable, an intermediate vaccine can be used in combination with an inactivated oil-emulsion product. Although costly in terms of labor and vaccine, this approach has proven effective for small units. Procedures relating to storage, reconstitution and administration of vaccines should be rigorously controlled.
CHAPTER TWO
MATERIALS AND METHODS

2.1. Preparation and sterilization

2.1.1. Preparation and sterilization of glassware
   Glassware's like flasks, beakers and measuring cylinders were rinsed in running tap water, brushed with special tissue culture flask soap, and left overnight in 1% HCl. Washed thoroughly by rinsing several times in tap and distilled water. After that they were wrapped with aluminum foil and sterilized in hot air oven at 160°C for 1 hour. The volumetric pipettes were left overnight in 3% HCl. Then they washed thoroughly by rising several times in tape and distilled water. The clean dry pipettes were cotton plugged, placed in canisters and sterilized in hot air oven at 160°C for 1 hour.

2.1.2. Preparation and sterilization of plastic wares
   Rubber liners for bottles and plastic cylinders were cleaned with detergent, washed with tap water followed by distilled water, left to dry and sterilized by autoclaving at 121°C (15lb/squire inch) for 15 minutes.

2.1.3. Preparation and sterilization of filters and filter papers
   Millipore filter papers (0.22 µ) were assembled in their appropriate holders, wrapped in aluminum foil and sterilized by autoclaving at 121°C (151b/squire inch) for 15 minutes.
2.2. Chicks

All chicks used in the present study (n=155) were kindly supplied by Coral Hatcheries, Khartoum. Chicks were obtained as day-old and reared till the required age.

2.3. Embryonated eggs

These were obtained from a white leghorn parent flock (Bovans breed). This flock is kept under strict hygienic measures at the premises of the Microbiology Department. Faculty of Veterinary Medicine, University of Khartoum.

2.4. IBD Strains

Two vaccines commercially available were selected for the study;

a- D78 inter mediate vaccine strain produced by intervet, Boxmear-Holland. Each does contain at least 4.0 log_{10} TCID_{50} of the Gomboro strain D78 plus stabilizers and antibiotics. Its application for vaccination is either by drinking water, spray and intranasal / intra-ocular routes.

b- Bur706 a modified live vaccine against IBDV produced by Merial Italia spa, Italy. Strain s706 has at least 10^{4} TCID_{50} plus stabilizers and antibiotics. Its application for vaccination is either by drinking water, spray and intranasal / intra-ocular routes.

C- Sudan local field isolate after inactivation.

2.5 IBD Challenge Virus

The live local field isolated in the work was used as the challenge virus.
2.6. Hyperimmuno serum

Three pair of rabbit was inoculated subcutaneously with D78 vaccine as IBDV antigen then after two weeks rabbit were given a double dose and another double does after three weeks and three weeks post last does blood was collected from the rabbit heart, in sterile test tubes. The blood was left for 2 hours at room temperature then the clot was loosened from the surface of the tubes and kept overnight at 4°C. Then the serum was separated and clarified by centrifugation at 2000 rpm for 10 minutes. The serum antibody was tested against IBDV antigen in AGPT.

2.7. The AGPT

2.7.1. Preparation of agar for AGPT:
On a glass container 1.4 g purified agar was added to 100 ml agar buffer (Appendix n=10) mixed and put in a steamer for an hour and then dispensed in Petri dishes in 17 ml volume per dish. The agar was solidified by air at room temperature. After solidification 7 holes were made, 6 outer and one inner. The 7 holes make a shape of circle. The diameter of each hole was 6 mm with 3 mm interspaces between holes.

2.7.2. Test procedure
Using a pipette 20 µL of 10% bursal or spleen homogenates were put in one of the 6 around wells and 20 µL of the hyperimmune serum was put in the inner well. Then the gel was incubated in a humidified chamber at room temperature for 24 – 48 hours
2.8. Chick embryo cell culture

2.8.1. Tissue culture preparation

To prepare chick embryo fibroblast monolayer ten-day-old embryonated eggs were used.

Procedure

Embryonated eggs were obtained from the poultry farm of Virology Research laboratory, Department of Microbiology, Faculty of Veterinary Medicine, University of Khartoum. Eggs were incubated till 9-11 day old. The incubated eggs were candled for viability and then the shell surfaces were cleaned by immersing in 70% alcohol for 5 minutes. The round shell just below air sac was cut by pairs of scissors, and the embryo was collected by placing the scissors under its head and the embryo lifted out.

The head and the limbs of the embryo were cut, viscera were removed and remains were fragmented with scissors into small fragments.

Then the fragments were washed several times with phosphate diluents (PD) (Appendix n=2) and transferred to a trypsinization flask.

The trypsinization was carried out with discontinuous manner. 5ml of prewarmed trypsin versin solution (Appendix n=5) per embryo were added and stirred slowly for 15 minutes at 37°C. After the tissue had settled out, the supernatant cells (containing single cells
and small cluster of cells) were collected in container with one to two ml of calf serum (Appendix n=6) and kept on ice. This was repeated 3-4 times until only the white fibrous tissues were left and no more cells dispense. Then the supernatant containing the pelleted cells were resuspended by the equation of each 1 cubic centimeter cells to 100ml of growth media and mixed by pipetting. Then the diluted suspension was distributed into 50 ml flask, 7.5 ml/flask and in 6 well plates (2 ml/well) and then incubated at 37°C. Confluent monolayers were established within 2-3 days.

2.9. ELISA for antibodies detection

Description of the Test

The IBD ELISA kit used was manufactured by BioChek, Gouda, Holland. The test kit was used according to the manufacturer instructions. Microtitre plates have been pre-coated with inactivated IBD antigen. Chicks’ serum samples are diluted and added to the microtitre wells where any anti-IBD antibodies present will bind and form an antigen–antibody complex. Non specific antibodies and other serum protein are then washed away. Anti-chicks IgG labeled with the enzyme alkaline phosphatase is then added to the wells and binds to any chicks anti–IBD antibodies bound to the antigen. After another wash to remove unrelated conjugate, substrate was added in the form of pNPP chromogen. A yellow colour is developed if anti-IBD antibody is present and the intensity is directly related to the amount of anti-IBD present in the sample.
2.9.1. Reagents provided

1. **IBD Coated plates.** Inactivated viral antigen on microtitre plates.

2. **Conjugate reagent** Anti-chicks: Alkaline Phosphatase in tris buffer with protein stabilizers, inter red dye and sodium azide preservative (0.1%w/v).

3. **Substrate tablets.** PNPP (p-Nitrophenyl Phosphate) tablets to dissolve with Substrate buffer.

4. **Substrate buffer reagent.** Diethanolamine buffer with enzyme co-factors.

5. **Stop solution.** Sodium Hydroxide in Diethanolamine buffer.

6. **Sample diluent reagent.** Phosphate buffer with protein stabilizers and sodium azide preservative (0.1%w/v).

7. **Wash buffer.** Powdered Phosphate buffer Saline with tween.

8. **Negative control.** Specific Pathogen Free serum in Phosphate buffer with protein stabilizers and sodium azide preservative (0.1%w/v)

9. **Positive control.** Antibodies specific to IBD in Phosphate buffer with protein stabilizers and sodium azide preservative (0.1%w/v)

2.9.2. Test procedure

1. IBD coated plate was removed from sealed bag and recorded location of samples on template.

2. 100µl of negative of negative control was added into wells A1 and B1.

3. 100µl of positive control was added into wells C1 and D1.
4. 100µl of diluted samples was added into the appropriated wells. Plate was covered with lid and incubated at room temperature for 30 minutes.

5. Wells contents were aspirated and washed 5 times with wash buffer (300µl per well). Plate inverted and tap firmly on absorbent paper.

6. 100µl of Conjugate reagent into the appropriated wells. Plate was covered lid and incubated at room temperature.

7. Wash was repeated as in step 5 procedure.

8. 100µl of Substrate reagent was added into the appropriated wells. Plate covered with lid and incubated at room temperature for 15 minutes.

9. 100µl of Stop solution was added to appropriate wells to stoped the reaction.

10. Then the microtitre plate was read at 405nm using an ELISA reader (Sun Raise) reader.

2.9.3. Interpretation of results

Samples with a sample to positive ratio (S/P) of 0.2 or greater contain anti-IBD antibodies and are considered positive.

Calculation of S/P ratio:

\[
\text{Mean of Test Sample - Mean of negative control = S/P}
\]

\[
\text{Mean of positive control - Mean of negative control}
\]
**Calculation of Antibody Titre**

The Following equation relates the S/P at a 1:500 dilution to an end point titre.

\[
\log (S/P) + 3.361. \ast \log_{10} \text{Titer} = 1.1
\]

\[\text{Antilog} = \text{Titer}\]

<table>
<thead>
<tr>
<th>S/P value</th>
<th>Titre Range</th>
<th>Antibody status</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.149 Or less</td>
<td>284 or less</td>
<td>Negative</td>
</tr>
<tr>
<td>0.150-0.199</td>
<td>285-390</td>
<td>Suspect</td>
</tr>
<tr>
<td>0.200 or greater</td>
<td>391 or greater</td>
<td>Positive</td>
</tr>
</tbody>
</table>

2.10. **Inactivated Vaccine development**

2.10.1. **Virus isolation 2.10.1.**

2.10.1.1. **Infectious bursa disease virus field isolate**

**Isolation of IBDV field isolate**

IBDV-field strain was isolated from an outbreak, which occurred at Shambat in Khartoum State in August 2006. The affected layer birds were 7 weeks old. The main clinical signs were depression, ruffled feathers, watery diarrhea. Morbidity and mortality rates were 100% and 90% respectively. Gross lesions were dehydrated carcasses; hemorrhage in thigh and pectoral muscles and at the junction between proventriculus and gizzard. Bursa of Fabriciuus was edematous, enlarged and some of them were hemorrhagic.

2.10.1.2. **Collection of tissue specimens**

Postmortem examination was performed and bursa and spleen were removed aseptically from infected chickens into sterile bottles.
and immediately transported in ice to the laboratory where the specimens stored at -20°C until used.

2.10.1.3. Preparation of tissue homogenate

The bursa were cut into small fragments using two scissors, and small amount of normal saline containing 1000 units /ml penicillin was added to make 40% homogenate by grinding the tissue fragment with a pestle and mortar. The homogenate was clarified by centrifugation at 3000 rpm for 10 minutes in a bench centrifuge. The supernatant was harvested, stored at – 20°c in sterile bijous and labeled.

2.10.2. Identification of IBDV antigen
2.10.2.1. By the AGPT.

Procedure of the test

Using a pipette, the test antigen was dispensed into the 6 outer wells while dispensing the hyperimmuno serum in the center one. The gel was then incubated in a humidified chamber at room temperature for 48 hours.

2.10.2.2. By virus isolation in chick embryo fibroblast

The bursal and spleen homogenates were inoculated onto chick embryo fibroblast. Each 25ml small cell culture flask received 0.5ml of the inoculums. The cell culture flasks were incubated at 37°c for 1 hour after which the inoculum was removed and the cell sheet washed with PD (Appendix n=2) three times. Maintenance media (Appendex n=12) was added and the flasks were incubated at 37°c. The flasks
were daily examined by an inverted microscope to check for cytopathic effect (CPE).

2.10.2.3. By experimental infection

A number of 15 six weeks old chicks were inoculated orally with the 1ml of the tissue homogenate. Additional group of 5 birds were left as uninfected control. The infected chicks were observed daily for clinical signs and the morbidity and mortality recorded. Postmortem examination was performed on recently dead chicks and specimens (bursa) for histopathology were taken.

2.10.3. Virus propagation and adaptation

Tissue homogenates were inoculated onto confluent chick embryo cell culture prepared in 25 cm$^3$ tissue culture flask. Each flask received 0.5 ml of the inoculum and left for 1 hour at 37°C for absorption. Then the inoculum was removed and the confluent monolayer were washed twice with PD (Appendix n=2) and refer with maintenance medium (GMEM) (Appendix n=12) and kept at 37°C. The cell culture was examined daily microscopically for the present of cytopathic effect (CPE). All the flasks that show CPE were harvested by repeated freeze-thaw cycles, and the suspension is clarified by low-speed centrifugation (1500 rpm for 10 minutes).The supernatant fluid was harvested.

2.10.4. Titration of IBDV Field Isolate in cell Culture

Ten fold serial dilutions $10^{-1}$ to $10^{-10}$ of the pooled IBDV field isolate harvests were prepared and used in titration procedure. CEF prepared in 96 wells microtiter plates were inoculated with the
diluted virus volume 0.1ml, each virus dilution was inoculated in five wells and at last five wells were left as controls and incubated for absorption for one hour then media was added. Daily investigation for cytopathic effect was done and the last reading was taken at the 5th day. The titer was calculated according to the method of Reed and Muench (1938).

2.10.5. Inactivation

Inactivation of the vaccine virus and any potential contaminants in the harvested virus was achieved by treatments with formalin. (Formaldehyde solution (37-41% w/v HCHO) LOBA CHEMIE PVT LTD Mumbai, India). The formalin was in concentration of 0.2% (v/v) (Habib et al., 2006) in a conical flask that contained a stirring bar. The mixture was stirred continuously and the inactivation was continued for 48 hours at room temperature. The inactivated vaccine was inoculated into 3-weeks old susceptible chicks at the rate of 0.3ml/bird subcutaneously (Helmboldt and Garner, 1964). Birds were observed for 5 day post inoculation.

2.10.6. Addition of adjuvant to the inactivated virus

Aluminum hydroxid (Rona Care™ Merck KgaA/ 64271 Darmstadt, Germany) was used as an adjuvant with the percentage 5%.

2.11. Experimental Vaccination Trials

A number of 140 layer chickens were divided into 7 groups of 20 chicks each.

Group1: Vaccinated with D78 in the drinking water according to the manufacturer instructions
Group 2: Vaccinated with Bur706 in the drinking water according to the manufacturer instructions.

Group 3: Vaccinated with the developed inactivation IBD vaccine Shambat/o4/adj. Each bird received 0.5 ml inoculated in the thigh muscle with 1ml disposable syringe and needle.

Group 4: Vaccinated with the developed inactivation IBD vaccine Shambat/o4/. Each bird received 0.5 ml inoculated in the thigh muscle with 1ml disposable syringe and needle.

Group 5: Vaccinated with the developed inactivation IBD vaccine Shambat/o4/adj. Each bird received 0.5 ml inoculated sub cut with 1ml disposable syringe and needle.

Group 6: Vaccinated with the developed inactivation IBD vaccine Shambat/o4. Each bird received 0.5 ml inoculated sub cut with 1ml disposable syringe and needle.

Group 7: unvaccinated control birds.

At day six of age a number of 25 sera were collected and examined by ELISA for antibodies against IBD to determine the best day of vaccination in which the maternal antibody allows. The first dose of vaccination was given at 17 days old according to the result. Another booster dose was given 10 days after the first dose for both developed inactivated vaccine and the two commercials vaccine (D78 and Bur706). Five weeks later sera were collected and tested for immune response by ELISA.
2.12. Challenge

All birds in the 7 groups were challenged with the local isolate of IBDV at the age of 5 weeks. Each bird received a dose volume of 0.5ml by the orally route that contained $10^{5.2}$ TCID$_{50}$/ml. The challenge virus was isolated in CEF and given at the second passage.
Chapter Three

Results

3.1. Detection of infectious bursal disease virus antigen in bursal tissues collected from field specimens

3.1.1. AGPT results

IBDV was identified in the 40% bursal homogenates. The sample gave precipitin line with the known antiserum against IBDV indicating the presence of IBDV antigen in the bursal homogenate (Fig.2).

3.1.2. Isolation of IBDV in CEF cell culture

The field isolate virus after tested with AGPT was inoculated onto chicken embryo fibroblast (CEF) cell culture. No cytopathic effect (CPE) was seen in the four days post inoculation. At the 5th day clear rounded cells were observed and the cells start to detach from the flask surface within 24 hours making small sized plaques distributed in different parts of the monolayer (Figures 1 and 2).

3.1.3. Titration of field isolate in CEF cell culture

The field isolate pool harvest (Shambat/06) gave a titer 5.2 log10 TCID50/0.1ml.

3.1.4. Experimental infection with IBDV field isolate.
A number of 15 chicks were inoculated with the bursal homogenate.

3.1.4.1. Clinical Signs

Typical clinical signs of IBD were observed at day 5 pi. The affected birds showed dullness, ruffled feather reluctant to move, recumbence with the beak on the earth off food and pasted vent. Mortality rate was 100%, at the same time no clinical signs were observed at the control group.

3.1.4.2 Postmortem findings

Experimental infected chicks showed dehydration of carcose, hemorrhages in skeletal muscles (thigh) (Figures 6, 7). In some cases there were hemorrhages in the junction of proventriculus and gizzard (Figure 8). Bursa of fabriciues enlarged (2-3 times) and became edematous, yellowish and in few birds hemorrhagic. No postmortem changes were observed in the control group

3.1.4.3 Histopathological lesions

The bursa of Fabricius of infected chickens showed and infiltration of mononuclear cells which consisted of lymphocytes. The lymphoid follicles showed degeneration. Hemorrhages, edema was occasionally observed in the connective tissues and between the dissociated follicular cells.

3.2. Inactivation result

The experimental chicks showed neither sign nor lesion after inoculated with the inactivated vaccine.
The inactivated vaccine was inoculated into 3-weeks old susceptible chicks at the rate of 0.3ml/bird subcutaneously. Birds were observed for 5 day post inoculation (Helmboldt and Garner, 1964).

3.3. Results of Immunity test

Antibody titer was detected by ELISA in sera of chicken vaccinated by both the commercials (D78 and Bur706) and the inactivated vaccine (Shambat/06).

For Bur706, D78 and Shambat/06 which administrated I/M and S/C with adjuvant or with out, table (1) express the comparative between them for the immune response and the challenge and the Figures from 8 to 13 expressed the group's immune results.
Figure 1. Wet preparation of uninfected chick embryo fibroblast cell culture 3 days after culturing
Figure 1-2. Wet preparation of fibroblast cell culture field infected after inoculated with field IBDV isolate shambat/06 at day 5\textsuperscript{th} shown cytopathic effect of cell rounding
Figure 2. Identification of IBDV by AGDT. Wells 1-4, 40% bursa homogenate. Well 5 control negative (Normal saline) well 6 control positive (Known IBDV antigen).
Figure 3-1 postmortem changes in chicks experimentally inoculated with bursa homogenate at day 6 pi. Arrow point to hemorrhage at the thigh muscles 6 days pi (arrow).
Figure 2-2. Postmortem changes in chicks experimentally inoculated with bursa homogenate at day 6 pi. Arrows shown hemorrhage in thigh muscles 6 days pi (arrow).

Figure 4. Proventriculus and gizzard collected at day 6 pi chicks inoculated with bursa homogenate. Arrow points to hemorrhages in the mucosa at the junction of the proventriculus and gizzard (arrow).
**Figure 5.** Section of bursa of fabricious collected from chicks experimentally inoculated with 40% bursa homogenate IBDV. Note; hemorrhage in the connective tissues and between the dissociated follicular cells (arrow).
Figure 6. Section of bursa of fabricsious collected from chicks experimentally inoculated with 40% bursa homogenate IBDV. Note, edema in the follicle of bursal of fibricius (arrow).
Figure 7. Section of bursa of fabricious collected from chicks experimentally inoculated with 40% bursa homogenate IBDV. Note, degeneration of follicles (arrow).
Table 1: Immune response and challenge of chicks vaccinated against IBD with 6 vaccination regimes.

<table>
<thead>
<tr>
<th>Protective immune response</th>
<th>Sh/06/adj I/M</th>
<th>Sh/06 I/M</th>
<th>Bur706 During water</th>
<th>D78 During water</th>
<th>Sh/06/adj S/C</th>
<th>Sh/06 S/C</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>14/15 = 93.3%</td>
<td>11/15 = 73.3%</td>
<td>14/15 = 93.3%</td>
<td>14/15 = 93.3%</td>
<td>7/15 = 46.7%</td>
<td>5/15 = 33.3%</td>
<td>0/20 = 0</td>
<td></td>
</tr>
<tr>
<td>With standing challenge</td>
<td>15/20 = 75%</td>
<td>13/20 = 65%</td>
<td>16/20b = 80%</td>
<td>15/20 = 75%</td>
<td>9/20 = 45%</td>
<td>7/20 = 35%</td>
<td>0/20 = 0</td>
</tr>
</tbody>
</table>

Sh= Shambat
Adj= adjuvant
Table 2: Distribution of positive ab level of chicks sera collected after vaccinated with Shambat/06 with and without adjuvant, D78 and Bur706.

<table>
<thead>
<tr>
<th>Ab range</th>
<th>Shambat/06/adj number of chicks (I/M)</th>
<th>Shambat/06/noadj number of chicks (I/M)</th>
<th>D78 vaccinated chicks number</th>
<th>Bur706 vaccinated chicks number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2000-4000</td>
<td>3</td>
<td>7</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>4000-6000</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>6000-8000</td>
<td>3</td>
<td>3</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>8000-10000</td>
<td>7</td>
<td>3</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>10000-12000</td>
<td>1</td>
<td>-</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>
**Figure 8.** Distribution of antibody titers groups in sera of chick's antibody titer group detected by ELISA after vaccinated using D78.

![Bar chart showing distribution of antibody titers groups in sera of chick's antibody titer group detected by ELISA after vaccinated using D78.](image)

- **Sera samples**
- **Titer group**
- 0=Negative results
Figure 9. Distribution of antibody titer groups in sera of chicks detected by ELISA after vaccinated using Bur 706 orally.

0=Negative results
**Figure 10.** Distribution of antibody titers groups in sera of chicks detected by ELISA after vaccinated by Shambat/06 without adjuvant S/C.

0=Negative results
**Figure 11.** Distribution of antibody titers groups in sera of chicks detected by ELISA after vaccinated by Shambat/06 with adjuvant S/C.

0=Negative results
Figure 12. Distribution of antibody titers groups in sera of chicks detected by ELISA after vaccinated by shambat/06 no adjuvant I/M.

0=Negative results
**Figure 13.** Distribution of antibody titers groups in sera of chicks detected by ELISA after vaccinated by shambat/06/adj I/M.
CHAPTER FOUR
DISCUSSION

The infectious bursal disease virus was first known in the Sudan as a health problem since 1980, yet the first published field report of the disease dated back to 1982 (Shuaib et al., 1982). The virus has since been implicated in many economic losses in different parts of the country. In addition, the increase use of antibiotics to fight opportunistic (secondary) infection is a major concern to human health, if we consider the risk linked to the presence or residues in meat. In Sudan outbreaks of the disease cause high mortality rate despite vaccination. This may denotes a vaccination failure which might be due to emergence of a vvIBDV that is antigenically different from the virus in the vaccines used (Snyder, 1990). The vvIBD strains could establish infection in the presence of high level of maternal antibodies that were protective against classical strains as indicated by Chettle et al (1989) and cause up to 60%-100% mortality (Van den Berg et al., 1991).

In the present study an inactivated IBD vaccine were developed from a local field isolate of the virus. It is anticipated that the field virus would induce better immune response in vaccinated birds due to its close antigenic relationship. A field outbreak of the disease that occurred in 2006 in Khartoum state (Shambat) was investigated that caused 70% mortality even in vaccinated bird. IBDV was detected by AGID test, isolation in cell culture (CEF) and
reproduction of the disease in experimental chicks. The virus was isolated in CEF cell culture and identified serologically by AGDT. The virus was designed Shambat/06 and further propagated for two passages in cell culture and the TCID$_{50}$ was determined. Then the CEF cell culture harvest was inactivated using formalin and the inactivation tested used experimental chicks.

A total of 140 layer chicks were used to determine the efficacy and safety of the developed vaccine and to compare it with commercial IBDV vaccines D78, Bur706.

The results showed that when the bursa suspension was inoculated onto chick embryo fibroblast cell culture it induce clear cytopathic effects after 5 days of inoculation. Clinical signs observed after experimental infection of susceptible chicks were similar to the classical IBD clinical signs and were characterized by sudden onset of depression, dullness, ruffled feather and death. Theses signs are similar to those described in the early outbreaks of IBD by Cosgrove (1962) and also to those described by Parkhurst (1964). Tissues collected from experimental chickens were examined histopathologically. Bursal of fabricius showed hemorrhage in the connective tissues and the dissociated follicular cells beside edema in the follicle and degeneration of follicles. These findings agree with that of Okoye (1983), Lang et al (1987) and Lasher Shane (1994).

A volume of 100 ml of the inactivated virus was mixed with Aluminum hydroxide as adjuvant. Experimental chicks were vaccinated by the inactivated vaccine and for comparison additional chicks were vaccinated with D78 and Bur706 commercial IBD
vaccines. No signs or lesions of IBD were seen and vaccinated chicks remained healthy throughout the experiment, which means that the IBDV in the vaccine was completely inactivated by the formalin.

Two preparations were made for the vaccine, with and without Aluminum hydroxide adjuvant and two routes of vaccination; intramuscular (I/M) and subcutaneous (S/C) were tried. The best result in terms of immune response and withstanding challenge was obtained when the adjuvanted inactivated vaccine was given by the intramuscular route. Results showed also that the developed vaccine without adjuvant when given by I/M gave a relatively lower protective immune response of 73.3% comparing with 93.3% for the adjuvanted vaccine which mean that the absent of the adjuvant make loss of 20% at immune response.

The results also revealed similar protective immune response for D78, Bur706 vaccine with the developed adjuvanted vaccine when given by I/M route. On the other hand, the developed vaccine without adjuvant when given by I/M showed 20% lower protective immune response compared with the commercial vaccines. The results also showed similler withstanding challenge for both shambat/06/adj and D78 while Bur706 was slightly better than both above vaccines. The higher uniformed higher titer from 8,000 upto 12,000 was induced in vaccinated chicks by the developed vaccine with adjuvant when given by I/M.

The efficacy of the developed vaccine observed in the present study is promising and indicate that the developed vaccine may became a good alternative method to control IBD in Sudan. Similarly
Hossain et al (2004) found that a local field isolate of infectious bursal disease virus in Bangladesh can induce higher immune response in chickens than that of the commercially available IBDV vaccines. The authors explained the reason as due to degradation of the quality of the imported vaccines during transportation or in a new environmental condition or due to antigenic dissimilarities among the local field virus and the imported vaccines.

To control IBD by vaccination farmers use live or inactivated vaccine despite the field situation and which vaccine is better at the particular time.

Advantages of killed-type vaccines are assurance of administration of a uniform dose (birds are individually injected), safety (the organism has been inactivated), development of uniform levels of immunity (each bird receives the same dose), no chance for spread of vaccine organism to neighboring poultry farms, increased product stability, and a choice of a wider variety of virus strains. Disadvantages are increased costs (labor and product), slower onset of immunity, narrower spectrum of protection, and presence of localized tissue damage at site of injection due to reaction with the adjuvant.

Further research is needed to study the properties of the local isolates of IBDV using molecular techniques. There is also a need to determine immune response of the developed inactivated vaccine in parent stocks for the protection of offspring chicks in broilers. Trials of inactivation using binary ethylenimine are also needed to compare the inactivation and immune response with that of formalin.
CONCLUSION

1- It is possible to isolate and propagate or produce local IBDV isolate in CEF cell culture, for diagnosis and vaccine production.

2- The developed inactivated vaccine induced good immune response and withstanding challenge comparable to two commercially available vaccines (D78, Bur706).

3- The Aluminum hydroxide as adjuvant increased remarkably immune response of chicks vaccinated with the inactivated IBD significantly.

4- The developed inactivated IBD with adjuvant vaccine administered I/M gave the highest numbers of protected birds and uniform antibody titer that ranged from 8,000 to 12,000, while the commercial vaccines gave less uniform antibody titer.
RECOMMENDATION

1- CEF Cell culture is recommended to be method of identification, isolation and propagation of IBD samples from the field.

2- Further research is needed to determine immune response of the developed inactivated vaccine in parent stocks for the protection of offspring chicks in broiler chicks.
REFERENCES


Azad, A. A; Barret SA and Fahey K. J. (1985). The characterization and molecular cloning of the double stranded RNA genome of an Austrain strain of IBDV. Virology 143: 36-44


APPENDIX

Reagents and solution

1-Normal saline (NS):

Stock solution of 0.85 % (w/v) NaCl was prepared in DDW and autoclaved at 121°C for 20 minutes.

Phosphate buffer saline (PBS).

Solution A:

NaCl 16gm
KCl 0.4gm
Na₂HPO₄ 2.3gm
KHPo₄ 0.4gm

Complete to 1500 ml by DDW

Solution B:

Hydrous MgCl₂6H₂O 0.426gm

Or

Anhydrous MgCl₂ 0.2gm

Complete to 200 by DDW
Solution C:

CaCl anhydrous 0.2gm

Or

CaCl$_2$H$_2$O hydrous 0.264gm

Complete to 200ml

Each solution was autoclaved separately and solution A and B were added to C, then was completed to 2litres by adding sterile DDW.

2- Preparation of phosphate diluents (PD):

Solution A of PBS was completed to 2liters with DDW; it was then autoclaved and cooled before antibiotic were added.

3-Preparation of Stock Trypsin (2.5%):

2.5 grams of trypsin (Gibcol td UK 1:250 Usp Grade) were dissolving in 100ml of PD, filtered through What Man filter and stored at -20°C.

4-Preparation of Stock Version (5%):

5 grams of versin powder was dissolved in 100ml PD, autoclaved at 121°C for 15 minutes.
5-Preparation of Trypsin Versin solution:

6ml of trypsin (2.5%) was added to 4ml of version (5%), completed to 100ml with phosphate diluents, with addition of few drops of phenol red were added pH adjusted by NaOH.

6-Bovine serum:

Calves were bled from the jugular vein. The whole blood was left overnight at room temperature serum was centrifuged at 2000 rpm for 10 minutes, and then filtered through Seitz filter under negative pressure, tested for sterility using thioglycolate media and kept at -20°C.

7-Preparation of antibiotic solutions:

One gram of streptomycin powder and the contents of 2 vails of penicillin (1000000 IU/vail) were dissolved in 10ml of sterile DDW so that 1ml of the prepared solution contained 100mg streptomycin and 2000000IU penicillin. The solution was kept at -20°C.

8-Mycostatin:

One vail of Mycostation (50000ug) was dissolved in ten ml of sterile DDW so that 1ml of the solution contained 5000ug.
9-Preparation of Thioglycolate medium:

Twenty nine and half gram of thioglycolate medium was dissolving and dispensed in bijoux bottle. The medium was sterilization by autoclaving at 121°C for 10 minutes.

10-Preparation of 500 ml buffer for IBD AGPT

Nacl 40.0gm

Phenol 2.5gm

DD.w make to 500 ml

Ph was adjusted to 7.5 by adding 1ml to 3ml NaoH solution

11- Preparation and sterilization of media and solutions:

To prepare the five fold stock solution, 125.19gm of Glasgow Modified Eagles Medium (GMEM) powder were dissolved in two liters of deionized distilled water (final volume). The solution was filtered through Millipore filter (0.22 u) under positive pressure, tested for sterility using thioglycolate medium and stored at -20°C.

12- Growth and Maintenance media:

GMEM X 5 concentration 200ml

0.5% lactalbumin hydrolysate 25ml

1% yeast extracts 25ml
Tryptose phosphate broth 25ml
7.5%NaHco₃ 8ml
Penicillin / streptomycin 1ml
Fungizone 1ml

DDW was added to complete to one liter. For preparation of growth media 100 ml of calf serum were added, 50 ml for maintenance.